SPERMIATION IN *RANA P. PIPIENS* IN RESPONSE TO HETEROPLASTIC PITUITARY MATERIALS

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Introduction

The earliest studies employing pituitary materials as a gonadal stimulant were conducted on mammals (Ascheim, 1926; Smith, 1926; Zondek, 1926). An excellent summary of vertebrate ovulatory responses induced by homoplastic and heteroplastic pituitary materials is provided by Creaser and Gorbman (1939). Amphibian spermiation was first evoked by a hormonal stimulus when Galli-Mainini (1947) reported the male toad, *Bufo arenarum,* responding to human chorionic gonadotropin. Since that date world-wide usage of the male anuran pregnancy test has involved numerous representative species from the families Discoglossidae, Pipidae, Ranidae, Microhylidae, Bufonidae, and Hylidae.

In view of the success of the male anuran pregnancy test it is surprising that the effect of pituitary gonadotropins upon spermiation has been little studied. In 1954 Houssay summarized some limited work on induced spermiation, reporting that spermiation is produced in *Bufo arenarum* by the pituitary glands of *Bufo arenarum, Leptodactylus ocellatus, Rana pipiens, Xenopus laevis, Bufo paracnemis, Bufo marinus,* and *Bufo d'Orbigny.* Reciprocally, Houssay found the anterior lobe of pituitary glands from *Bufo arenarum* to produce spermiation in *Leptodactylus ocellatus* and *Bufo marinus.* Crude extracts from pituitary glands of ox, sheep, swine, dog, guinea pig, cat, and fishes did not produce spermiation in *Bufo arenarum.* Limited positive results were reported using high doses of pituitary extracts from rat, rabbits, and man. Pure follicle-stimulating hormone and luteinizing hormone from sheep pituitary also gave positive results. Burgos and Ladman (1955) reported sperm release by *Rana pipiens* after the administration of purified swine follicle-stimulating hormone, sheep luteinizing hormone, and human chorionic gonadotropin. Witschi and Chang (1958), summarizing all available data on spermiation and ovulation reactions, put spermiation on the same basis with ovulation, though stating the former to be a more sensitive reaction.

The present study seemed justified since a very spotty and in-

complete representation from the vertebrate series had been studied in relation to the induced spermiation reaction. The purpose of the present investigation was therefore, to observe the effect of representative vertebrate pituitary materials upon spermiation in the male northern leopard frog, *Rana p. pipiens.*

Materials and Methods

A detailed species list of all pituitary donors employed in this study is given in Table I. The giant tree frogs (*Hyla septentrionalis*), Key anoles (*Anolis stejnegeri*), and sharks (*Sphyrna tiburo, Carcharhinus platyodon*) were collected from the Key West area. The tropical marine toad (*Bufo marinus*) was taken from a recently introduced population now breeding in the Miami area. Except for the mudpuppies (*Necturus maculosus*), Louisiana specimens ordered from the Carolina Biological Supply Company, the remaining species were collected in Volusia County, Florida or obtained locally (chickens, white mice). Lederle Laboratories generously supplied frozen swine whole pituitaries. The recipient species, *Rana p. pipiens,* were ordered in one-dozen lots from the Carolina Biological Supply Company.

Pituitary glands were removed from each donor as soon as it was sacrificed. In the majority of cases the pituitary was removed as follows: the head was severed from the body at the base of the occipital bone and the lower jaw was then removed. From the remaining cranium, the membranes covering the dorsal roof of the mouth were removed. This exposed the ventral bones of the cranium, in particular the basioccipital, basisphenoid, and presphenoid complexes. The ventral floor of the brain case was then removed by inserting scissors or Liston bone-cutting forceps into the foramen magnum, lateral to the brainstem, and snipping anteriorly. The pituitary was usually easily distinguished, lying immediately posterior to the optic chiasma, on the infundibular region of the hypothalamus. The gland was removed with fine-pointed watchmaker's forceps and placed into 2 ml. of amphibian ringer's solution. If the pituitary was not to be used immediately, it was frozen until time for injection. It has been shown that no adverse effects are encountered using pituitary materials that have been frozen for periods up to one year (Hansen, 1955).

Whole glands from donors were thoroughly macerated with a small mortar and pestle to facilitate the injection. The fresh

or frozen gland macerate was mixed thoroughly in 2 ml. of amphibian ringer solution and then injected subcutaneously with a 25 gauge needle. To prevent leakage, the needle was inserted into the dorsal lymph sac and then directed to either side into the two lateral lymph sacs. Any leakage from the lateral lymph sacs would, therefore, move through the connective tissue back into the dorsal lymph sac. No leakage was ever noted at the point of insertion on the dorsum.

Urine was obtained from the recipient frogs from two to four hours after injection. Cloacal fluid was taken by aspiration, by inserting a small, blunt-tipped pipette into the external cloacal orifice. A few drops of the urine were placed on a microscope slide and examined under low magnification for the presence of spermatozoa. The presence of sperm indicated a positive test, its absence, a negative test.

Because of the possibility of natural spermiation, each frog used in the study was checked for the presence of sperm in the cloacal fluid just prior to injection. In no case was any sperm ever found. Natural emission of sperm in anura seems to be rare as no cloacal sperm were found even when animals were collected from a breeding population (Hansen, 1960). Although individual refractoriness by a test animal can never be ruled out completely, multiple injections in the majority of cases probably negates this possibility to a large extent. Further controls used throughout the study were as follows: injection of amphibian ringer's solution, human male urine, human pregnancy urine, and solutions of macerated frog brainstem. Negative results were obtained in all cases except for the pregnancy urine which induced spermiation.

A number of the test animals were re-used after negative and positive tests, although a recovery period of not less than five days was allowed in both cases. This period seemed adequate in the light of Burgos and Ladman's report (1955) where spermiating *Rana pipiens* were re-used after 48 hours, with no observed cumulative effect.

Results

This study demonstrated the effect of representative vertebrate pituitary homogenates, specifically the gonadotropic hormones, upon the spermiation reaction in the male northern leopard frog, *Rana p. pipiens*. These results are listed in Table I.

TABLE I

EFFECT OF PITUITARY INJECTIONS UPON SPERMIATION IN RANA P. PIPIENS

Pituitary Donor	No. Pituitary Glands Injected	Size(s) of Donor(s)	Spermiation Result
CHONDRICHTHYS		Total length (head-tail, cm.)	
Sphyrna tiburo	1 male	137	neg.
Carcharhinus platydon	1 male	132	neg.
OSTEICHTHYS		Standard length (head-caudal fin base, cm.)	
Lepisosteus osseus	2	49,52	pos.
Esox niger	2	54,69	neg.
Micropterus salmoides floridanus	5	35,38,40,43,47	neg.
AMPHIBIA		(Head-tail, mm.)	
Necturus maculosus	1 female	265	neg.
	2 "	260,262	neg.
	3 "	249,258,261	pos.
		(Snout-vent, mm.)	
Scaphiopus h. holbrooki	1 male	62	neg.
	2 "	58,59	neg.
	3 "	55,57,60	pos.
	1 female	65	neg.
	2 "	53,57	pos.

TABLE I—Continued

EFFECT OF PITUITARY INJECTIONS UPON SPERMIATION IN RANA P. PIPIENS

Pituitary Donor	No. Pituitary Glands Injected	Size(s) of Donor(s)	Spermiation Result
Rana p. pipiens	1 male	63	pos.
	1 female	88	pos.
Rana p. sphenocephala	1 male	71	pos.
	1 female	79	pos.
Rana catesbeiana	1 male	129	pos.
	1 female	132	pos.
	.5 "	156	pos.
	.25 "	156	pos.
Rana grylio	1 male	145	pos.
	.5 female	151	pos.
	.25 "	151	pos.
Bufo marinus	1 male	137	pos.
Bufo t. terrestris	1 male	68	neg.
	2 "	48,55	pos.
	1 female	80	pos.
Hyla septentrionalis	3 male	51,54,58	pos.
	2 female	65,69	pos.
Hyla squirrela	3 female	27,30,31	pos.

TABLE I—Continued

EFFECT OF PITUITARY INJECTIONS UPON SPERMIATION IN RANA P. PIPIENS

Pituitary Donor	No. Pituitary Glands Injected	Size(s) of Donor(s)	Spermiation Result
REPTILIA		(Carapace length, mm.)	
Sternotherus odoratus	1 male	74	neg.
	1 female	88	pos.
Deirochelys reticularia	1 female	112	neg.
Pseudemys floridana peninsularis	1 male	218	neg.
	1 "	239	neg.
	1 female	308	pos.
Gopherus polyphemus	1 male	147	neg.
	1 "	270	neg.
	1 female	210	neg.
	1 "	240	pos.
Tryonx f. ferox	1 female	225	pos.
		(Snout-vent, mm.)	
Anolis c. carolinensis	1 male	50	neg.
Anolis stejnegeri	2 male	47,55	neg.
Cnemidophorus sexlienatus	11 individuals (7 males, 4 females)	68,69,69,70,71,72 73; 55,65,68,70	pos.
		(Total length, cm.)	
Coluber constrictor priapus	1 female	107	neg.
Coluber f. flagellum	1 female	137	neg.
Drymarchon corais couperi	1 male	139	neg.
Elaphe obsoleta quadrivittata	1 male	152	neg.

TABLE I—Continued

EFFECT OF PITUITARY INJECTIONS UPON SPERMIATION IN RANA P. PIPIENS

Pituitary Donor	No. Pituitary Glands Injected	Size(s) of Donor(s)	Spermiation Result
AVES			
Gallus gallus	2 female	8 wks.	pos.
	4 "	1 year	pos.
	6 "	8 wks.	pos.
Cyanocitta cristata	2 sex undetermined	adult	pos.
MAMMALIA			
Myotis austroriparius	1 male	adult	neg.
Felis domesticus	1 female	adult	neg.
	.5 "	"	neg.
	.25 "	"	neg.
Sus scrofa	2 sex undetermined	adult	pos.
	1 " "	"	neg.
	.3 " "	"	neg.
	.1 " "	"	neg.
	.05 " "	"	neg.
Sciurus c. carolinensis	3 male	adult	neg.
Mus musculus	1 female	adult	neg.
	3 "	"	neg.
	6 "	"	neg.

Pituitary homogenates from two elasmobranchs, the bonnethead shark (*Sphyrna tiburo*) and Gulf shark (*Carcharhinus platydon*) failed to evoke spermiation in the experimental animals. Pituitary macerates from the long-nose gar (*Lepisosteus osseus*) caused sperm release, whereas, two teleost species (Chain pickerel, *Esox niger;* Florida largemouth bass, *Micropterus salmoides floridanus*) failed to do so.

From the Amphibia, representative species from four anuran families responding positively included: the eastern spadefoot, *Scaphiopus h. holbrooki*; northern leopard frog, *Rana p. pipiens;* southern leopard frog, *Rana pipiens sphenocephala;* southern bullfrog, *Rana grylio;* American bullfrog, *Rana catesbeiana;* southern toad, *Bufo t. terrestris;* marine toad, *Bufo marinus;* squirrel tree frog, *Hyla squirrela;* and giant tree frog, *Hyla septentrionalis.* From the Urodela, the mudpuppy (*Necturus maculosus*) also effected a positive response.

Spermiation responses due to pituitary materials from the Reptilia proved somewhat variable. Chelonian donors evoking a positive response included large-sized representatives from four families; the southern soft-shelled turtle (*Tryonx f. ferox*), mud turtle (*Sternotherus odoratus,* peninsular turtle (*Pseudemys floridanus peninsularis*) and gopher tortoise (*Gopherus polyphemus*). A negative response was obtained from the chicken turtle (*Deirochelys reticularia*). Single pituitary homogenates from four snakes (indigo, *Drymarchon corais couperi;* coachwhip, *Coluber f. flagellum;* Florida black snake, *Coluber constrictor priapus;* rat snake, *Elaphe obsoleta quadrivittata*) and two lizards (Carolina anole, *Anolis c. carolinensis;* Key anole, *Anolis stejnegeri*) failed to stimulate spermiation. A pituitary homogenate containing eleven pituitaries from the six-lined racer (*Cnemidophorus sexlineatus*) did evoke a positive response.

Multiple pituitary homogenates from two avian species (chicken, *Gallus gallus;* bluejay, *Cyanocitta cristata*) caused *Rana p. pipiens* to spermiate.

Representatives from four mammalian orders were tested and no positive results were realized from the smaller mammals, including the Florida brown bat (*Myotis austroriparius*), laboratory mice (*Mus musculus*), southern grey squirrel (*Sciurus c. carolinen-*

sis) and domestic cat (*Felis domesticus*). The only positive result came from hog (*Sus scrofa*) pituitary in which two whole glands were necessary to induce the reaction.

Discussion

Creaser and Gorbman (1939) ably demonstrated in their induced ovulation studies that a qualitative species specificity exists within vertebrate gonadotropic hormones. The results of the present work bear out this generality in that some vertebrate pituitary materials readily evoke anuran spermiation while others do not. The variation in the present results can almost certainly be attributed to phyletic differentiation in the pituitary gonadotropins as well as to hormonal amount and titer.

It seems almost certain that some of the negative reactions may be attributed to the small amounts of pituitary material available from the smaller species as the snakes, lizards, bat, and mice. This reasoning seems verified in a number of instances (*Bufo, Scaphiopus, Necturus, Cnemidophorus*) where small amounts of pituitary failed to induce sperm release, while larger amounts did evoke a positive response.

It seems reasonable to assume that the anuran endocrine system would have differentiated to a higher degree than specialized members of lower vertebrate groups, as the teleosts. The fact that pituitary material from the Chondrichthys and Osteichthys, with the exception of the long-nose gar (*Lepisosteus osseus*), failed to induce spermiation, lends validity to the hypothesis of species specificity.

Perhaps the most interesting problem arising in this study was the positive response obtained by pituitary from the long-nose gar. This case was the sole exception to total negative responses by pituitary materials from all vertebrates phylogenetically below the Amphibia. Creaser and Gorbman report:

> "Wills, Riley, and Stubbs have demonstrated a positive ovulation inducing capacity of *Lepisosteus* (gar pike) pituitaries in the toad *Bufo americanus* and in one specimen of *Rana pipiens*. The later datum deserves checking and should prove of considerable significance if verified, since Lepisosteus is phylogenetically more closely related to the amphibian forms than any of the fishes studid so far."

The present finding, therefore, adds validity to this earlier work and indicates an endocrine relationship between the anura and the ancestoral holostean group.

The positive reaction obtained with the mudpuppy, *Necturus maculosus,* is the first record of caudate amphibian pituitary acting positively upon the gonads of an anuran species. This is not in agreement with the induced ovulation study upon anuran species (Creaser and Gorbman, 1939). Induced spermiation reactions by reptile, bird, and mammal pituitary preparations are in general agreement with Creaser and Gorbman (1939) and Hansen (1959) in their ovulation studies.

It seems noteworthy that somewhat larger amounts of pituitary material from donor species outside the Ranidae are needed to cause sperm release. The need for two entire hog pituitary glands to induce spermiation is in keeping with Creaser and Gorbman's findings. These workers stated, "Those amphibia in which a response is produced by mammalian preparations require very large doses". Because of variations in pituitary size and gonadotropic titers, it is apparent that an accurately quantified work in this general area is seriously needed. The primary difficulty presently is that no standard method for determining accurate hormone titer has as yet been forthcoming.

In several cases (*Bufo, Gopherus, Pseudemys*), it was noted that a single female pituitary would cause a positive reaction while a single male pituitary from the same species would not. In *Scaphiopus* where a multiple pituitary homogenate was used, two male glands failed to evoke a response, whereas two female glands did so; three male glands did bring about the reaction. This quantitative evidence corroborates the findings of Rugh (1937) and Hansen (1959), that the female pituitary has a higher hormone titer than that of the male of the same species.

Summary

1. Pituitary-induced spermiation tests were conducted from October, 1961 through May, 1962 on 66 northern leopard frogs, *Rana p. pipiens.*

2. Fresh pituitary preparations from 111 individuals representing 34 vertebrate donor species were used in the study.

3. Spermiation was induced by pituitary materials from representatives of the Osteichthys, Amphibia, Reptilia, Aves, and Mammalia, while negative reactions resulted from the Chondrichthys.

4. The female pituitary gland was found to have a higher potency than the male gland in evoking spermiation.

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